Specific Ribosomal DNA Marker for Early Polymerase Chain Reaction Detection of *Aphelinus hordei* (Hymenoptera: Aphelinidae) and *Aphidius colemani* (Hymenoptera: Aphidiidae) from *Diuraphis noxia* (Homoptera: Aphididae)

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ABSTRACT To monitor aphid parasitism by *Aphelinus hordei* (Kurdjumov) and *Aphidius colemani* Viereck, we developed specific ribosomal DNA markers to distinguish them from several other cereal aphid parasitoid species and two important host species, the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), and the greenbug, *Schizaphis graminum* (Rondani). Ribosomal DNA sequences for the internal transcribed spacer 2 (ITS2) were first cloned and sequenced from *A. hordei*, *A. albipodus* Hayat & Fatima, *A. asychis* Walker, *A. varipes* (Foerster), *A. colemani*, *D. noxia*, and *S. graminum*. We designed specific primers based on the ITS2 sequences. Polymerase chain reaction (PCR) amplification of wasp and aphid DNA using these primers, followed by agarose gel electrophoresis, successfully distinguishes *A. hordei* and *A. colemani* from all three other *Aphelinus* species and two aphid species. A 411-bp nucleotide fragment and a 571-bp fragment were amplified only from *A. hordei* and from *A. colemani*, respectively, and no such fragments were amplified from any other wasp species or aphids. DNA could be detected at a level as low as 10^{-3} adult wasp equivalent for *A. hordei* and 5×10^{-4} adult wasp equivalent for *A. colemani*. The DNA of both species was detectable in parasitized *D. noxia* 24 h after initial contact with adult parasitoid pairs.

KEY WORDS *Aphelinus hordei*, *Aphidius colemani*, *Diuraphis noxia*, biological control, parasitoid, polymerase chain reaction

THE ACCURATE IDENTIFICATION of natural enemies is critical to the success of classical biological control programs, particularly in determining the fate and evaluating the performance of newly introduced species (Delluchi et al. 1976). One of the most intensive classical biological control programs of recent decades has been the campaign against the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), in the United States (Hopper et al. 1998, Prokrym et al. 1998a). A fifth of the introduced taxa resulting from this effort were hymenopterous parasitoids in the genera *Aphelinus* (Aphelinidae) and *Aphidius* (Aphidiidae). One species, *Aphelinus albipodus* Hayat & Fatima, was a new introduction and was successfully established (Prokrym et al. 1998b).

One aphelinid that was not part of the U.S. Russian wheat aphid campaign is *Aphelinus hordei* (Kurdjumov), a species that was introduced into South Africa in 1991 for Russian wheat aphid control (Prinsloo 1998). Here we describe the development of DNA markers for *A. hordei* and for the aphidiid *Aphidius colemani* Viereck, two species recently released at sites in the U.S. southern Great Plains (J.D.B. and N.C.E., unpublished data).

Materials and Methods

Insects. Protocols for rearing aphids and parasitoids have been described elsewhere (Reed et al. 1991). They were maintained in cages in a Conviron model 123 incubator (Controlled Environments, Pembina, ND), at 20°C and a photoperiod of 16:8 (L:D) h. Founding stocks for colonies of *A. asychis* from Antibes, France, and of *A. varipes* from Montpellier, France, were provided by K. R. Hopper of the USDA–ARS Beneficial Insects Introduction Research Unit in Newark, DE; *A. albipodus* from Tacheng, Peoples Republic of China, were provided by D. Gonzáles of the University of California, Riverside; *A. hordei* from Bethlehem, South Africa, were provided by G. J. Prinsloo of the Small Grain Institute, Bethlehem. The *A. hordei* population was descended from material collected at Odessa, Ukraine (Prinsloo and Neser 1994). To reduce the risk of contamination, only one *Aphelinus* colony was maintained at one time. Our *A. colemani* stock, descended from material collected in La Cruz, Region V, Chile (Starý 1993), was obtained from K. S. Pike of Washington State University.

Diuraphis noxia and another important cereal aphid, the greenbug *Schizaphis graminum* (Rondani), were

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Table 1. Primer sequences and fragment size produced in PCR amplification

Name	Sequence	Temp[a]	Target species	Fragment size, bp
58S-F	TGTGAACTGCAGGACACATGAAC	50	*A. albipodus*	531
28S-R	ATGCTTAAATTTAGGGGGTA		*A. asychis*	528
			A. varipes	533
			A. hordei	531
			A. colemani	721
Aho-FAC	ATGAACAGACTGCTCGCGTC′	63	*A. hordei*	411
ITS2-R	AGTCTCGCCTGCTCTGAGGT′			
Aco-F	CTAGCGATAAATGAATGTTCGTA	60	*A. colemani*	571
28S-R	ATGCTTAAATTTAGGGGGTA			

[a] Optimal annealing temperature, °C.

from colonies maintained at the USDA-ARS Plant Science Research Laboratory in Stillwater, OK. The aphids were reared on wheat. All insect populations used in this research had been in colony for three or more years.

DNA Extraction, Cloning, and Sequencing of Ribosomal ITS2. Genomic DNA was isolated from 5–10 individuals of both aphids and wasps, without regard to sex, as previously described (Zhu and Greenstone 1999). Following RNase A digestion at a final concentration of 20 g/ml for 30 min at 37°C, the DNA solution was extracted once with one volume of chloroform/isoamyl alcohol (24:1). DNA was precipitated with two volumes of ethanol overnight at −20°C, pelleted by centrifugation, and resuspended in 200 μl of distilled water.

The DNA fragment was amplified using the forward primer 58SF and reverse primer 28SR (Table 1) of Porter and Collins (1991). PCR conditions, agarose gel electrophoresis, and extraction of PCR products were as described earlier (Zhu and Greenstone 1999). Isolated DNA fragments were ligated into a pGEM-T vector (Promega, Madison, WI) overnight at 14°C, and the ligation solution was used to transform *Escherichia coli* cells. Transformed cells were plated on LB/ampicillin/IPTG/X-Gal medium. White colonies were subjected to PCR amplification to confirm the presence of inserts and the expected sizes of fragments. PCR-confirmed colonies were inoculated into 25 ml LB/ampicillin cultures, and plasmid DNA was extracted using a Qiagen Plasmid Midi kit (Qiagen, Santa Clarita, CA). DNA inserts were sequenced from both directions using an automated sequencer located at the Recombinant DNA/Protein Resource Facility, Oklahoma State University, Stillwater. For each species, 1–2 clones were sequenced.

Primer Design and PCR Amplification of Wasp and Aphid DNA. After ITS2 DNA sequences were obtained from all wasp and aphid populations, GCG UNIX version nine (Genetics Computer Group, Madison, WI) was used to align and analyze the sequences. The detection technique for single-base substitution (Kwok et al. 1990) was used to design primers for separating *A. hordei* and *A. colemani* from one another and from *D. noxia* and *S. graminum.* Five individuals of each wasp population and each aphid species were subjected to individual DNA extraction and PCR amplification. PCR reactions were set up as described earlier (Zhu and Greenstone 1999), and were performed in a PTC-100 thermocycler (MJ Research, Watertown, MA). DNA was initially denatured for 3 min at 94°C, and the PCR amplification was conducted for 60 cycles, with 30-s denaturing at 94°, 30-s annealing at 63°C for *A. hordei* or 60°C for *A. colemani*, and 1-min extension at 72°C. PCR products (10 μl) were separated on a 1% agarose gel, stained with 0.5 g/ml ethidium bromide, and photographed under UV light.

Detection Sensitivity. To determine the sensitivity of the PCR reaction to wasp DNA concentration, DNA of *A. hordei* or *A. colemani* was serially diluted five times from 10^{-2} to 10^{-5} adult wasp equivalent in a constant concentration of 10^{-2} Russian wheat aphid equivalent DNA in water. Wasp DNA without aphid DNA and aphid DNA without wasp DNA were included as controls. PCR reactions (20 μl) containing appropriate primers for *A. hordei* or *A. colemani* were amplified for 60 cycles using the appropriate annealing temperature for each wasp species as described above, and 20 μl of PCR product was subjected to gel electrophoresis.

Parasitization Detection. To obtain parasitized aphids, groups of 60 *D. noxia* were exposed to 25 pairs of adult *A. hordei* or *A. colemani.* Aphids were collected at 24 h and 48 h after initial exposure to wasps. For each interval, genomic DNA of 18 aphids was individually extracted, and DNA was PCR-amplified with wasp DNA only as positive control and aphid DNA as negative control. Twenty μl of PCR reaction containing appropriate wasp DNA and primers was amplified using the conditions described above, and 20 μl of PCR product was analyzed on agarose gel for parasitoid DNA.

Voucher Specimens. Vouchers of *A. hordei* and *A. colemani* have been deposited in the USDA–ARS–PSWCRL Cereal Insect Genetics Resource Library, Stillwater, OK. Voucher information for the other aphelinid species was reported previously (Zhu and Greenstone 1999).

Results and Discussion

Ribosomal ITS2 Sequences. ITS2 fragments of approximately 530 bp were amplified from all *Aphelinus* species using primers 58SF and 28SR. The *A. hordei*

fragment was cloned and a 531-bp nucleotide sequence resulted; a 721-bp fragment was cloned and sequenced from *A. colemani* (Table 1; Fig. 1). Sequences were confirmed as ribosomal ITS2 by similarity searching the GenBank database of the National Center for Biotechnology Information (Altschul et al. 1990; Gish and States 1993) with the BLASTn protocol.

The ITS2 sequence of *A. hordei* has a slightly higher A/T (adenine and thymine) bias (54%). The ITS2 sequence of *A. colemani* has a very high A/T bias (80%). Distribution of cytosine is not uniform along the *A. colemani* nucleotide strand, and no cytosine was found between position 210–300 and 551–625. There is <5% guanine from position 151–350, and no guanine from position 251–325.

Specific Primers and DNA Markers for *A. hordei*. Based on sequence alignment of the ribosomal ITS2 DNA sequences of all parasitoids and the two aphids, primers Aho-FAC and ITS2-R (Table 1) were designed to separate the wasps from *S. graminum* and *D. noxia* and from one another. Because of high identity of the ITS2 sequences in some regions, the single-base detection technique (Kwok et al. 1990) was applied to design Aho-FAC primer specific for *A. hordei* only by placing a nucleotide C at the 3′ end of the primer to create a mismatch between the primer and most other wasp ITS2 DNA templates. With Aho-FAC and another primer based on a conserved sequence alignment on the 28s ribosome gene, ITS2-R (Table 1), we expected a 411-bp fragment to be amplified from *A. hordei* exclusively.

PCR amplification was performed using these specific primers and DNA prepared from all parasitoid and aphid species. The 411-bp DNA fragment was consistently amplified from all five individual *A. hordei* DNA preparations (Fig. 2A, lanes 1–5). There was no corresponding amplification of a 411-bp fragment from pooled DNA of five individuals of *A. colemani* (lane 6), nor from pooled DNA of five individuals of *A. albipodus* (lane 7), *A. asychis* (lane 8), *A. varipes* (lane 9), S. *graminum* (lane 10), or *D. noxia* (lane 11). This experiment was repeated with another batch of insects, and the 411-bp fragment was again reproducible from the DNA template of *A. hordei* only.

Specific Primers and DNA Marker for *A. colemani*. Primer Aco-F was designed on the basis of the unique ITS2 sequence of *A. colemani* to separate it from the *Aphelinus* and aphid species when used with primer 28SR (Table 1). We expected a 571-bp fragment to be amplified from *A. colemani* only.

Polymerase chain reaction amplification was performed with these primers and DNA prepared from all wasp and aphid species. A 571-bp DNA fragment was consistently amplified from DNA of all five individual *A. colemani* (Fig. 2B, lanes 1–5), but no such fragment was amplified from pooled DNA of five individuals of *A. hordei* (lane 6), nor from pooled DNA of *A. albipodus* (lane 7), *A. asychis* (lane 8), *A. varipes* (lane 9), *S. graminum* (lane 10), or *D. noxia* (lane 11).

Sensitivity of PCR Amplification. To determine DNA template sensitivity, a variable amount of wasp DNA was added to a constant concentration of *D. noxia* DNA to simulate a homogenate of a parasitized host. PCR amplification of DNA from *A. hordei* using primers Aho-FAC and ITS2-R yielded expected 411-bp fragments that could be visually differentiated from negative controls at and above a concentration of 10^{-3} adult *A. hordei* DNA equivalent (Fig. 3A). PCR amplifications were repeated with different batches of DNA, and the results were repeatable. In one replicate experiment, the lowest DNA concentration could be extended to 3×10^{-4} wasp equivalent.

Similar experiments were conducted using primers Aco-F and 28SR to amplify aphid DNA spiked with different amounts of *A. colemani* DNA. A 571-bp fragment was generated that could be visually differentiated from negative controls at and above a concentration of 5×10^{-4} adult *A. colemani* DNA equivalent (Fig. 3B).

These sensitivities are of the same order of magnitude as in our previous study (Zhu and Greenstone 1999). From these data we are confident that our current PCR system can detect as little as 10^{-3} adult wasp DNA equivalent, and it may be possible to detect even lower wasp DNA amounts in aphid DNA mixtures by adjusting PCR conditions or adding secondary PCR amplification.

Early Detection of Parasitization. DNA was extracted from 18 aphids each held for 24 and 48 h following exposure to adults of each wasp species. PCR amplification was performed using primers Aho-FAC and ITS2-R for *A. hordei* and Aco-F and 28SR for *A. colemani*.

Both species are detectable as early as 24 h after exposure to parasitoids, *A. hordei* as a 41-bp DNA fragment (Fig. 4A) and *A. colemani* as a 571-bp DNA fragment (Fig. 4B). No corresponding fragments were amplified from the nonparasitized aphid DNA controls. For *A. hordei*, seven of 18 aphids collected 24 h after initial exposure to wasps, and nine of 18 aphids collected 48 h after initial exposure to wasps, were determined as parasitized. For *A. colemani*, one of 18 aphids collected at 24 h after initial exposure to wasps, and six of 18 aphids collected at 48 h after exposure to wasps, were determined as parasitized.

These data show that we can detect parasitization early, but not whether we can do so quantitatively; that will require very large experimental comparisons of dissected and PCR-probed aphids (cf. Greenstone and Edwards 1998).

Detection of aphid parasitism is technically challenging because of the small size of both hosts and parasitoids, and because there are a large number of very closely related potential parasitoids attacking the same hosts (Hopper et al. 1998, Prokrym et al. 1998b). This publication and our previous one (Zhu and Greenstone 1999) bring to six the number of parasitoid species and strains that can be identified and detected within cereal aphid hosts by PCR. Such tools will become increasingly useful as new opportunities for biological control arise in cropping systems in which cereals are interspersed among crops that provide additional alternative hosts for parasitoids (Dhuyvetter et al. 1996, Holtzer et al. 1996, Peterson et al. 1996).

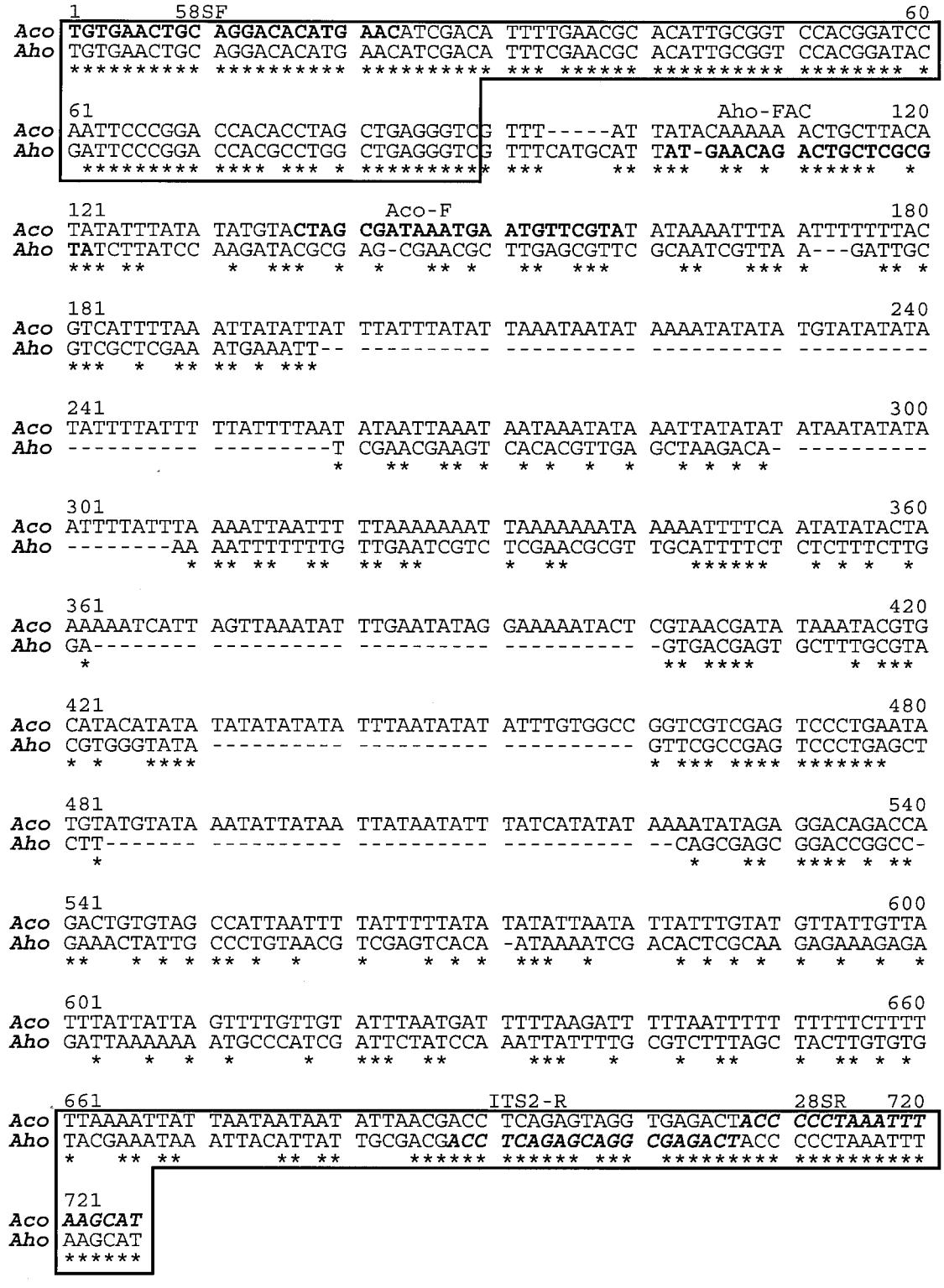

Fig. 1. Sequences of ribosomal internal transcribed spacer two (ITS2) of *A. colemani* (Aco) and *A. hordei* (Aho). Sequences for 5.8S and 28S ribosome units are boxed. Forward primer sequences are in bold case, and complementary reverse primer sequences are in bold italic case. Identical sequences are indicated by stars (*) below the sequences; hyphens represent sequence alignment gaps. A, adenine; C, cytosine; G, guanine; T, thymine.

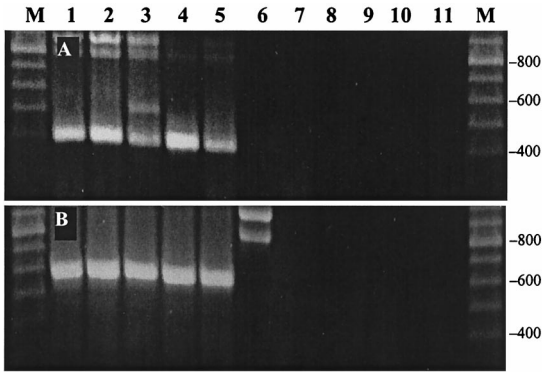

Fig. 2. (A) PCR amplifications for distinguishing *A. hordei* (411-bp fragment, lanes 1–5) from four wasp species, *A. colemani* (lane 6), *A. albipodus* (lane 7), *A. asychis* (lane 8), and *A. varipes* (lane 9); and two cereal aphids, *S. graminum* (lane 10) and *D. noxia* (lane 11), using primers Aho-FAC and ITS2-R. (B) PCR amplifications for distinguishing *A. colemani* (571-bp fragment, lanes 1–5) from four wasp species, *A. hordei* (lane 6), *A. albipodus* (lane 7), *A. asychis* (lane 8), and *A. varipes* (lane 9); and two cereal aphids, *S. graminum* (lane 10) and *D. noxia* (lane 11), using primer Aco-F and 28SR.

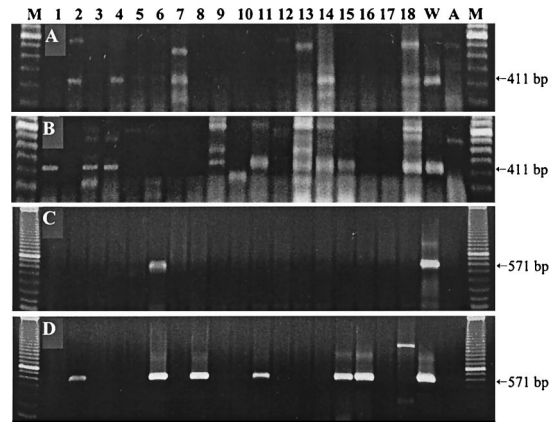

Fig. 4. PCR amplification for detecting *A. hordei* (lanes 1–18) within Russian wheat aphids collected 24 h (A) and 48 h (B) after initial exposure to adult wasps, and for detecting *A. colemani* within Russian wheat aphids collected 24 h (C) and 48 h (D) after initial exposure to adult wasps. (W) *A. hordei* DNA control in A and B and *A. colemani* DNA control in C and D; (A) *D. noxia* DNA control; M = 100-bp DNA marker from Pharmacia.

Furthermore, *D. noxia* and other cereal aphids continue to be a worldwide problem, and this same suite of parasitoid species will prove useful in many localities. Our goal is to have as complete a panel as possible of PCR primers for parasitoid natural enemies of the cereal aphid complex.

Aphidius colemani is widely distributed in Asia, southern Europe, Africa, South America, and Australia, and broadly oligophagous on aphids (Mackauer and Starý 1967, Starý 1975). Although its host range varies geographically (Stary 1975), it is known to parasitize several economically important aphid pests of cereals, including *S. graminum* and *D. noxia* (Mackauer and Starý 1967, Starý 1975, Aalbersberg et al. 1988). Our *A. hordei* stock descended from animals collected from *D. noxia* in Odessa, Ukraine (Aeschlimann and Hughes 1992) and appears to have adapted well to the same host in South Africa (Prinsloo 1998). We hope that both species will establish successfully in the U.S. southern Great Plains, an area similar climatically to the regions from which our stocks originated. If so, our PCR primers will be useful to document their progress as they find their place within the diverse assemblage of parasitoids already attacking cereal aphids there (Jackson et al. 1970, Jackson and Eikenbary 1971, Elliott et al. 1995).

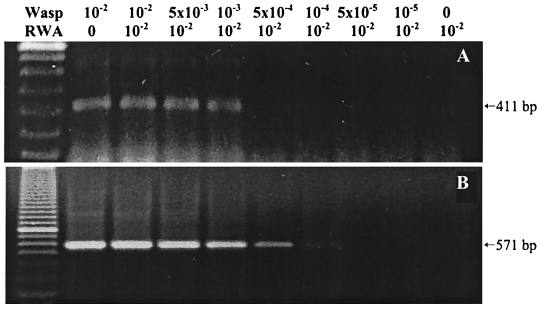

Fig. 3. PCR amplification of Russian wheat aphid DNA spiked with different amounts of *A. hordei* DNA (A) and *A. colemani* DNA (B). Wasp, amount of DNA in adult wasp DNA equivalent of each species; RWA, amount of DNA in adult aphid DNA equivalent of *D. noxia.* Left-most column is 100-bp DNA marker from Pharmacia.

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